

# Mucosal immunization of piglets with purified F18 fimbriae does not protect against F18<sup>+</sup> *Escherichia coli* infection

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## Abstract

Post-weaning diarrhoea and oedema disease in weaned piglets are caused by infection with F4<sup>+</sup> or F18<sup>+</sup> *Escherichia coli* strains. There is no commercial vaccine available, but it is shown that oral immunization of weaned piglets with purified F4 fimbriae induces a protective mucosal immune response. In the present study, piglets were orally and nasally immunized with purified F18 fimbriae in the presence of the mucosal adjuvant LT(R192G) or CTA1-DD, respectively. This immunization could not lead to protection against F18<sup>+</sup> *E. coli* infection. The induced F18-specific immune response was directed towards the major subunit FedA and weakly towards the adhesive subunit FedF. The results of these experiments demonstrate that it is difficult to induce protective immunity against F18<sup>+</sup> *E. coli* using the whole fimbriae due to the low response against the adhesin.

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## 1. Introduction

Newly weaned piglets are highly susceptible to F4<sup>+</sup> and F18<sup>+</sup> enterotoxigenic *Escherichia coli* (ETEC) and F18<sup>+</sup> verotoxigenic *E. coli* (VTEC) infections. The F4 and F18 fimbriae of these pathogenic *E. coli* are composed of major subunits and different minor subunits and allow adherence of the bacteria, respectively, to the F4 and F18 receptor (F4R and F18R) on small intestinal enterocytes (Jones and Rutter, 1972; Bertschinger et al., 1990; Meijerink et al., 2000).

However, the adhesive subunit of F4 fimbriae is the major subunit FaeG, whereas the adhesin of F18 fimbriae is the minor subunit FedF (Bakker et al., 1992; Imberechts et al., 1996; Smeds et al., 2003). Following colonization, ETEC strains produce one or more enterotoxins inducing secretory diarrhoea (Nataro and Kaper, 1998), whereas VTEC strains produce a vasotoxin resulting in edema and subsequent neurological signs (MacLeod and Gyles, 1991).

F18<sup>+</sup> *E. coli* are widely spread (Verdonck et al., 2003; Cheng et al., 2005) and are a major cause of economic losses in the pig industry due to diarrhoea, growth retardation and mortality. Several methods to prevent F18<sup>+</sup> *E. coli* infections were examined (Imberechts et al., 1997; Kyriakis et al., 1997;

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Tsiliyiannis et al., 2001; Nollet et al., 1999; Felder et al., 2000), but no vaccine or good therapy exists to date. The presence of F18 fimbriae-specific antibodies by passive immunization or active immunization, was reported to reduce F18<sup>+</sup> *E. coli* colonization (Bertschinger et al., 2000; Zuniga et al., 1997; Sarrazin and Bertschinger, 1997).

Previous experiments by our group revealed that oral immunization of weaned piglets with purified F4 fimbriae, induced an F4-specific systemic and mucosal immune response protecting these piglets against a subsequent F4<sup>+</sup> ETEC challenge (Van den Broeck et al., 1999a,b). The aim of the present study was to analyse whether mucosal immunization of piglets with purified F18 fimbriae could induce an active fimbriae-specific immune response, able to reduce the excretion of F18<sup>+</sup> *E. coli* following an F18<sup>+</sup> VTEC challenge. Piglets were orally or nasally immunized with purified F18 fimbriae in the presence or absence of the mucosal adjuvants LT(R192G) (Dickinson and Clements, 1995) or CTA1-DD (Agren et al., 1997). LT(R192G) is a mutated variant of LT that lacks the toxic activity of LT but remains its adjuvant effect. CTA1-DD is a fusion protein consisting of the enzymatically active A1 subunit of CT and two Ig-binding domains of staphylococcal protein A. This molecule has a strong ADP-ribosyltransferase activity and can bind to both Fc and Fab fragments of all classes of antibodies. This targeting of the adjuvant activity of CT (without its toxic activity) results in an improved expansion of activated B cells in the germinal center (Lycke, 2005).

## 2. Materials and methods

### 2.1. Pigs

Conventionally bred pigs (Belgian Landrace × Piétrain) were weaned at the age of 3 weeks (nasal immunization experiment,  $n = 10$ ) or 4 weeks (oral immunization experiment,  $n = 30$ ) and subsequently housed in isolation units, fed ad libitum and treated orally with colistine up to 3 days following weaning (Promycine pulvis, VMD, Berendonk, Belgium, 150,000 U/kg body weight/day) to prevent *E. coli* infections. The piglets were screened to be F18R<sup>+</sup> using the PCR method described by Meijerink et al. (1997) as well as F18-seronegative using the F18-specific ELISA (Verdonck et al., 2002). At the end of the experiments, the presence of the F18R was confirmed using the *in vitro* villous adhesion assay (Van den Broeck et al., 1999c). Adhesion of more than five bacteria per 250  $\mu\text{m}$

villous length was noted as positive (Cox and Houvenaghel, 1993).

### 2.2. Purification of F18 fimbriae and production of the adhesin FedF

F18 fimbriae were purified as described by Verdonck et al. (2002). Briefly, bacteria of the F18<sup>+</sup> VTEC strain F107/86 were collected from overnight cultures, whereafter F18 fimbriae were isolated by heat shock (60 °C for 20 min) and larger fragments were removed by two centrifugation steps (20 min 10,000  $\times g$  and 40 min 20,000  $\times g$ ). The solubilized F18 fimbriae were precipitated with 20% (w/v) ammonium sulphate and the pellet was dissolved and dialysed overnight against ultra pure H<sub>2</sub>O and stored at –20 °C. The protein concentration of purified F18 fimbriae was determined using the bicinchoninic acid reaction with bovine serum albumin (BSA) as a standard (ICN Biomedicals, Belgium).

The FedF encoding sequence of strain F107/86 was cloned in the pET-30 Ek/LIC vector and expression of recombinant FedF was induced by adding 1 mM isopropyl- $\beta$ -D-thiogalactoside (IPTG, Sigma) to overnight cultures (Tiels et al., 2007). FedF was isolated from the insoluble cytoplasmatic fraction, further purified using a Ni-NTA kit (Novagen) and overnight dialysed against PBS at 4 °C. The protein concentration of recombinant FedF was determined using the bicinchoninic acid reaction with bovine serum albumin (BSA) as a standard (ICN Biomedicals, Belgium).

### 2.3. Experimental procedure

#### 2.3.1. Oral immunization

Thirty F18-seronegative, F18R<sup>+</sup> piglets were randomly divided into five groups of six animals each. One week post-weaning, four groups of piglets were orally immunized on 3 subsequent days (0, 1 and 2 days post-primary immunization, dppl) and again 14 dppl with 10 or 30 mg purified F18 in the absence (10 mg F18 group and 30 mg F18 group, respectively) or presence of 25  $\mu\text{g}$  LT(R192G) (Dickinson and Clements, 1995) (10 mg F18 + LT group and 30 mg + LT group, respectively). The antigen and adjuvant were diluted in PBS to a final volume of 5 ml. A group of six animals received PBS (PBS group) and served as negative control. Before immunization, the animals received orally 20 mg omeprazole (Losec, Astra Pharmaceuticals, Brussels) to neutralize the acidic gastric pH and to protect the antigens against possible denaturation (Snoeck et al., 2004). Three hours before till 2 h after

immunization, each animal was deprived of food and water.

One week following the booster immunization, the animals were challenged on 2 consecutive days (21 and 22 dppi) with the virulent F18<sup>+</sup> VTEC strain F107/86 (serotype O139:K12:H1, F18ab<sup>+</sup>, sLT-IIv<sup>+</sup>) as previously described (Verdonck et al., 2002). Briefly, the pigs were sedated with Stresnil (40 mg/ml; Janssen-Cilag, Berchem, Belgium), whereafter the gastric pH was neutralized by intragastrical administration of 62 ml NaHCO<sub>3</sub> (1.4% (w/v) in distilled water). After 15–30 min, the piglets were intragastrically inoculated with 10<sup>11</sup> F18<sup>+</sup> VTEC (in 10 ml PBS). Faeces were sampled daily from 2 to 7 days after the first inoculation (2–7 days post-challenge, dpc) for determining the faecal excretion of F18<sup>+</sup> *E. coli*. In addition, blood was sampled 0, 7, 14, 21, 24, 32 and 38 dppi for measuring the F18- and FedF-specific IgM, IgA and IgG response. LT(R192G)-specific serum antibodies were analysed at the moment of challenge. Two weeks following challenge, the animals were euthanized by intracardial injection of pentobarbital (24 mg/kg; Nembutal, Sanofi Santé Animale, Brussels, Belgium) and subsequent exsanguination. Jejunal villi were isolated to confirm expression of the F18 receptor.

### 2.3.2. Nasal immunization

Ten F18R<sup>+</sup>, F18-seronegative pigs were immunized nasally at 1 week of age, on 3 successive days (0, 1 and 2 dppi) with 1 mg purified F18 fimbriae in the absence (F18 group, *n* = 5) or presence of 100 µg CTA1-DD (Agren et al., 1997) (F18 CTA1-DD group, *n* = 5), and again at the moment of weaning (4-week old, 23–25 dppi). The F18 fimbriae and the adjuvant were diluted in 1 ml PBS and administered dropwise into both nostrils. One week post-weaning (30 and 31 dppi), the pigs were challenged with the virulent F18<sup>+</sup> VTEC strain F107/86 as described above. Faecal excretion of F18<sup>+</sup> *E. coli* was determined daily from 2 till 9 dpc. Furthermore, F18- and FedF-specific IgM, IgA and IgG were determined in serum at 0, 23, 30, 37, 39, 44 and 50 dppi. At the moment of challenge (30 dppi), cholera toxin A subunit-specific antibodies were analysed in serum and F18-specific IgM and IgA were analysed in saliva and nasal secretion. Euthanasia was performed 3 weeks following challenge (50 dppi) and jejunal villi were isolated to confirm expression of the F18 receptor.

### 2.4. Analysis of F18<sup>+</sup> *E. coli* excretion

F18<sup>+</sup> *E. coli* were enumerated in faecal samples by dot blotting as described by Verdonck et al. (2002), with

some modifications. In the present study, suspensions of faecal samples in PBS were spread onto plain blood agar plates containing 400 µg/ml streptomycin to select for the streptomycin resistant F18<sup>+</sup> *E. coli* strain F107/86 and incubated at 37 °C for 24 h. F18<sup>+</sup> *E. coli* were detected using the F18(FedA)-specific MAb IMM02 (Tiels et al., 2007) and a horseradish peroxidase (HRP)-conjugated rabbit anti-mouse immunoglobulin anti-serum (Dako, Prosan, Merelbeke, Belgium). The resulting brown-red dots were counted and the average within each group was calculated.

### 2.5. Detection of antigen-specific antibodies

Serum, saliva and nasal secretions were sampled as described (Van den Broeck et al., 1999b; Van der Stede et al., 2002), to determine antigen-specific serum and mucosal antibodies. Cholera toxin A subunit-specific antibodies were analysed as described by Akhiani et al. (2006).

To detect LT(R192G)-specific antibodies, the wells of a 96-well microtiter plate (NUNC, Maxisorp Immuno Plates; Life Technologies, Merelbeke, Belgium) were coated with 5 µg/ml LT(R192G) in PBS during 2 h at 37 °C. Subsequently, the plates were blocked overnight at 4 °C with 0.2% (v/v) Tween<sup>®</sup>80 in PBS. Serial 2-fold dilutions of serum samples (starting from 1/10) in ELISA dilution buffer (PBS + 0.05% (v/v) Tween<sup>®</sup>20 + 3% (w/v) BSA) were made and incubated for 1 h at 37 °C. Thereafter, an optimal dilution of HRP-conjugated rabbit anti-swine (Dako) was brought on the plate for 1 h at 37 °C. Finally, an ABTS solution containing H<sub>2</sub>O<sub>2</sub> was added and after 1 h incubation at 37 °C the optical density was spectrophotometrically measured at 405 nm (OD<sub>405</sub>). Between each incubation step, the plates were washed three times with ELISA washing buffer (PBS + 0.2% (v/v) Tween<sup>®</sup>20).

For detection of F18-specific antibodies, the indirect ELISA described by Verdonck et al. (2002) was used. Briefly, the wells of a 96-well microtiter plate (NUNC, Maxisorp) were coated with purified F18 fimbriae in carbonate–bicarbonate buffer (50 mM, pH 9.4). This coating with purified F18 fimbriae will mainly result in the detection of FedA-specific antibodies since purified F18 fimbriae contain 100–1000 times more FedA major subunits compared to the minor subunit FedF (unpublished data). After blocking of the remaining binding sites, serial dilutions of sera, saliva and nasal secretion were made in ELISA dilution buffer, starting from 1/10, 1/2 and 1/4, respectively. Subsequently, optimal dilutions of swine-specific IgM, IgA and IgG MAb

(Van Zaane and Hulst, 1987) and rabbit anti-mouse HRP-conjugated serum (Dako) were added. Finally, the OD<sub>405</sub> was spectrophotometrically measured.

FedF-specific antibodies were analysed in an ELISA identical to the F18 ELISA, except that the plates were coated with FedF (2 µg/ml in carbonate–bicarbonate buffer).

The cut-off values were calculated as the mean OD<sub>405</sub>-value of all sera (dilution 1/10) at day 0, increased with three times the standard deviation. The antibody titer was the inverse of the highest dilution that still had an OD<sub>405</sub> higher than the calculated cut-off value.

### 2.6. Statistical analysis

Statistical analysis (SPSS 10.0 for Windows) of F18- and FedF-specific antibody titers and F18<sup>+</sup> *E. coli* excretion was done using the General Linear Model (Repeated Measures Analysis of Variance), adjusting for multiple comparison by Bonferoni. Differences between groups in LT(R192G)-specific titer and CT-A-specific titer were analysed for statistical significance using the Mann–Whitney *U*-test.  $p < 0.05$  was considered as statistically significant.

## 3. Results

### 3.1. Oral immunization of piglets with F18 fimbriae

Primary oral immunization of weaned F18-seronegative and F18R<sup>+</sup> piglets with 10 or 30 mg purified F18 fimbriae (10 and 30 mg groups, respectively) resulted 2 weeks later (14 dppi) in the appearance of FedF adhesin-specific IgM, IgA and IgG serum antibodies in 3–4 animals per group (Fig. 1). However, these low antibody titers are not significantly higher than the background level of the PBS control group. In the 10 and 30 mg groups, antibodies against purified F18 fimbriae (mainly containing FedA subunits and only minor amounts of FedF adhesins) were first detected 1 week following the booster immunization (21 dppi).

Oral co-administration of purified F18 fimbriae with the mucosal adjuvant LT(R192G) (10 mg + LT and 30 mg + LT groups) was not able to clearly improve the induction of F18- and FedF-specific serum antibodies. At 21 dppi, only the mean F18-specific IgM titer of the 30 mg + LT group was significantly higher in comparison with the 10, 30 mg and PBS groups ( $p \leq 0.038$ ) (Fig. 1A). The lack of an improved induction of a fimbriae-specific antibody response is not due to inactive LT(R192G) since LT-specific total antibody

titers were significantly higher ( $p < 0.001$ ) in the LT(R192G)-supplemented groups (mean titers 320 and 452) at the moment of challenge (21 dppi) than in the non-supplemented groups where almost no LT-specific antibodies were detected (mean titers  $\leq 12$ ). Therefore, these results show that oral immunization of newly weaned piglets with purified F18 fimbriae in the presence or absence of the mucosal adjuvant LT(R192G) only weakly induces fimbriae-specific serum antibodies.

One week following the booster immunization (21 dppi), the piglets were challenged on 2 consecutive days (0 and 1 days post-challenge, dpc) with F18<sup>+</sup> VTEC and F18<sup>+</sup> *E. coli* excretion was determined daily (Fig. 2). Piglets of the negative control group (PBS group) excreted between  $2.2 \times 10^7$  and  $8.4 \times 10^8$  F18<sup>+</sup> *E. coli* per gram faeces at 2 dpc, which decreased to  $0$ – $8.2 \times 10^5$  F18<sup>+</sup> *E. coli* per gram faeces at 7 dpc. The excretion in the immunized groups was very similar, with only a non-significant reduction in the excretion in the 30 mg + LT group at 5 and 7 dpc. Nevertheless, these results indicate that oral immunization of newly weaned piglets with 10 or 30 mg purified F18 fimbriae in the presence or absence of LT(R192G) did not induce significant amounts of protective fimbriae-specific mucosal antibodies.

### 3.2. Nasal immunization of piglets with F18 fimbriae and CTA1-DD

A second experiment was performed to induce F18-specific mucosal and systemic antibodies at weaning. F18-seronegative and F18R<sup>+</sup> piglets were immunized nasally with purified F18 fimbriae in the absence or presence of the CTA1-DD adjuvant (F18 group and F18 CTA1-DD group, respectively) at 1 week of age and boosted at weaning. The nasal route was selected since uptake of milk during suckling can interfere with orally administered fimbriae and as a consequence interfere with the induction of a fimbriae-specific immune response. Furthermore, the CTA1-DD adjuvant has been shown to be effective in the nasal route (Akhiani et al., 2006; Dell et al., 2006). One week following booster immunization (30 dppi), low levels of IgM and IgG F18-specific and FedF-specific IgM serum antibodies were found in the F18 CTA1-DD group (Fig. 3). On the other hand, no F18- or FedF-specific serum antibodies could be detected in animals immunized with purified F18 without adjuvant. This difference can be related to the adjuvant effect of CTA1-DD. At the moment of challenge, all piglets of the CTA1-DD supplemented group had a CTA-specific serum titer

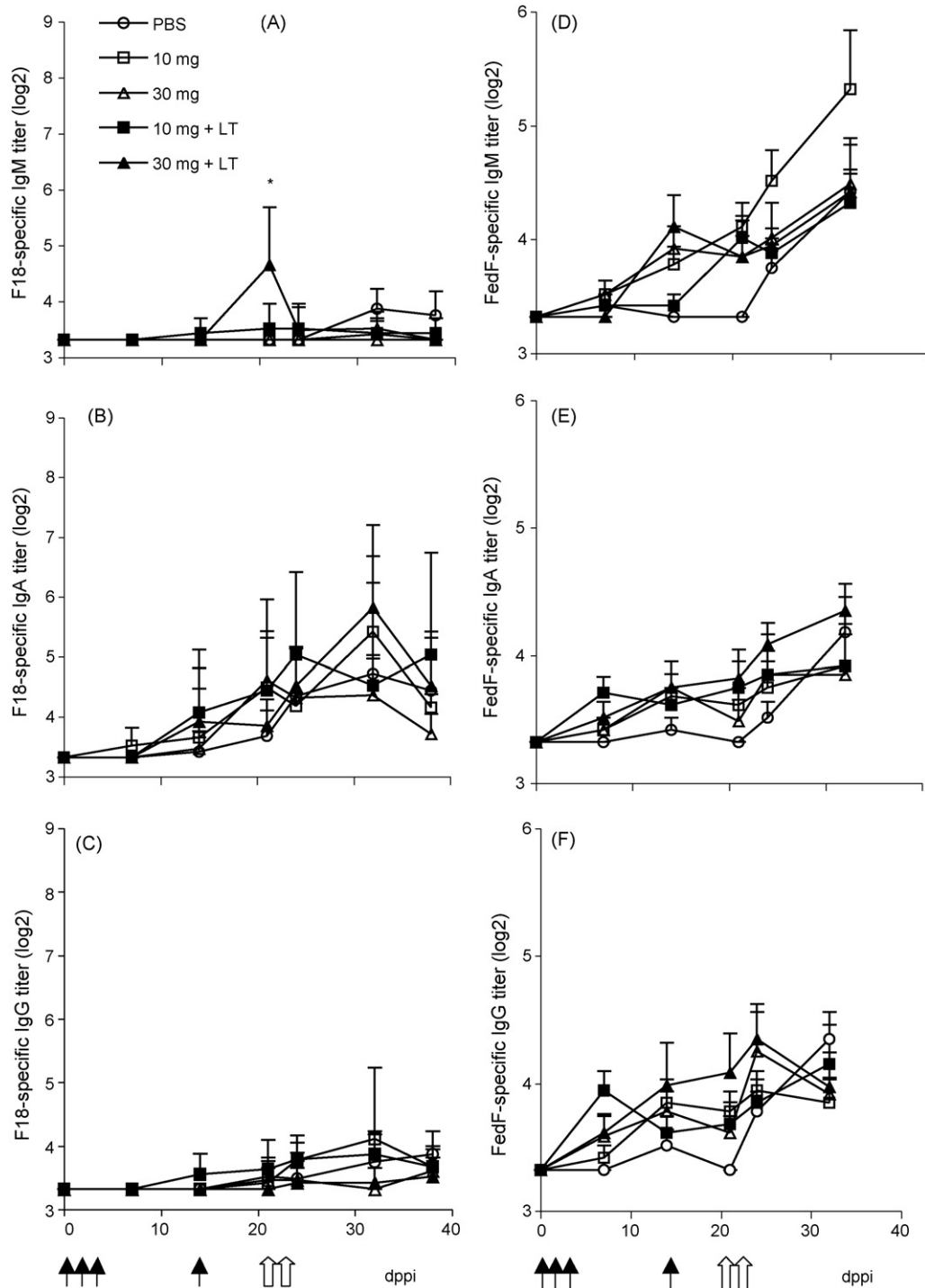


Fig. 1. Mean F18-specific IgM (A), IgA (B) and IgG (C) serum antibody titers ( $\pm$ S.E.M.) and FedF-specific IgM (D), IgA (E) and IgG (F) serum antibody titers ( $\pm$ S.E.M.) of piglets ( $n = 6$  per group) orally immunized with PBS (PBS group), 10 mg F18 (10 mg group), 30 mg F18 (30 mg group), 10 mg F18 + 25 µg LT(R192G) (10 mg + LT group) and 30 mg F18 + 25 µg LT(R192G) (30 mg + LT group). \*Significant difference ( $p < 0.05$ ) between 30 mg + LT group and PBS, 10 mg and 30 mg groups. Black arrow, immunization; white arrow, F18<sup>+</sup> VTEC challenge.



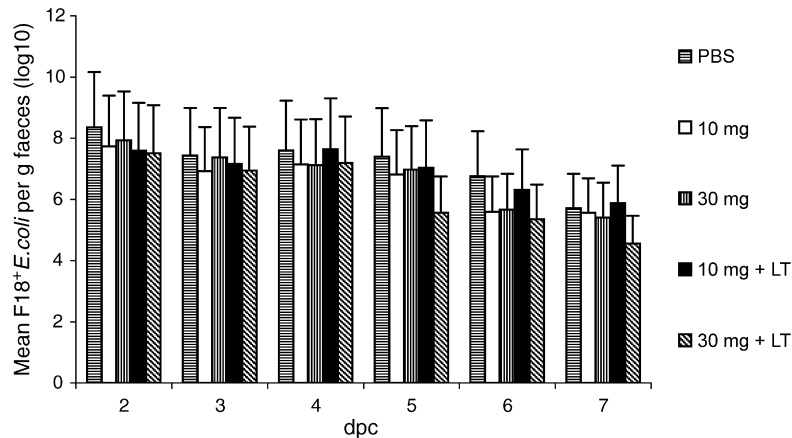


Fig. 2. Mean faecal F18<sup>+</sup> *E. coli* excretion (log 10) per gram faeces ( $\pm$ S.E.M.) of piglets ( $n = 6$  per group) orally immunized with PBS (PBS group), 10 mg F18 (10 mg group), 30 mg F18 (30 mg group), 10 mg F18 + 25  $\mu$ g LT(R192G) (10 mg + LT group) and 30 mg F18 + 25  $\mu$ g LT(R192G) (30 mg + LT group).

$\geq 160$  whereas the titers in the other group were  $\leq 20$  ( $p = 0.007$ ). In agreement with the F18-specific serum response, no significant differences were observed in IgA and IgM F18-specific antibodies in nasal secretions and saliva of the F18 and the F18 CTA1-DD groups (data not shown). However, nasal immunization of piglets with purified F18 fimbriae in the presence of CTA1-DD was able to prime a fimbriae-specific immune response. Indeed, 1 week following an F18<sup>+</sup> VTEC challenge (37 dpi), a significant higher F18-specific IgM antibody titer ( $p = 0.012$ ) was found in this group compared to the F18 group (Fig. 3A). Nevertheless, this priming could not lead to a reduction in excretion of F18<sup>+</sup> *E. coli* following challenge (Fig. 4).

#### 4. Discussion

In the present study, piglets were immunized mucosally with purified F18 fimbriae to induce fimbriae-specific antibodies, inhibiting adhesion of F18<sup>+</sup> *E. coli* to F18R<sup>+</sup> villi. Oral immunization of piglets with 1 mg purified F4 fimbriae was reported to induce a protective F4-specific immune response (Van den Broeck et al., 1999a). However, a preliminary experiment showed that oral immunization of piglets with 2 mg purified F18 fimbriae induced a non-protective F18-specific serum antibody response (titers  $< 22$ ) (unpublished data). Also oral immunization of piglets with microencapsulated purified F18 fimbriae induced no significant F18-specific serum antibodies (Felder et al., 2000). Several factors could account for this difference in immunogenicity between F18 and F4 fimbriae. A first factor could be the different structure of both fimbriae. The adhesin of F18 is the

minor subunit FedF (Imberechts et al., 1996; Smeds et al., 2003), whereas the adhesin of F4 fimbriae is the major fimbrial subunit FaeG (Bakker et al., 1992). Recent results of our group also suggest that the interaction between the FedF adhesin and the major subunit FedA is not as stable as subunit–subunit interactions in other fimbriae like F4, type 1 and P pili (Tiels et al., 2007). This may lead to a lower capacity of F18 fimbriae than F4 fimbriae to bind to their specific receptor on intestinal enterocytes, which may at least partly explain the slower and lower induction of a fimbriae-specific immune response following an F18<sup>+</sup> VTEC infection as compared with an F4<sup>+</sup> ETEC infection (Verdonck et al., 2002). Furthermore, the structural difference between both fimbriae perhaps influences their stability in the gastrointestinal tract. A second reason for the different immunogenicity of F4 and F18 could be related with different properties of the fimbrial receptor or its localization, but this has to be analysed in further experiments.

Due to the lower immunogenicity of F18 compared to F4, doses of 10 and 30 mg purified F18 fimbriae were used in the present study and a mutant of the heat-labile enterotoxin, LT(R192G), was used as adjuvant (Dickinson and Clements, 1995). Only 30 mg purified F18 fimbriae in the presence of LT(R192G), induced a significant higher F18-specific IgM serum response (21 dpi) and a low (non-significant) reduction in F18<sup>+</sup> *E. coli* excretion from 5 dpc onwards. The limited adjuvant effect of the LT(R192G) in the present study was not caused by an inactivity of LT(R192G) as high mLt-specific titers were found in the LT(R192G) immunized groups and not in the non-immunized group. This

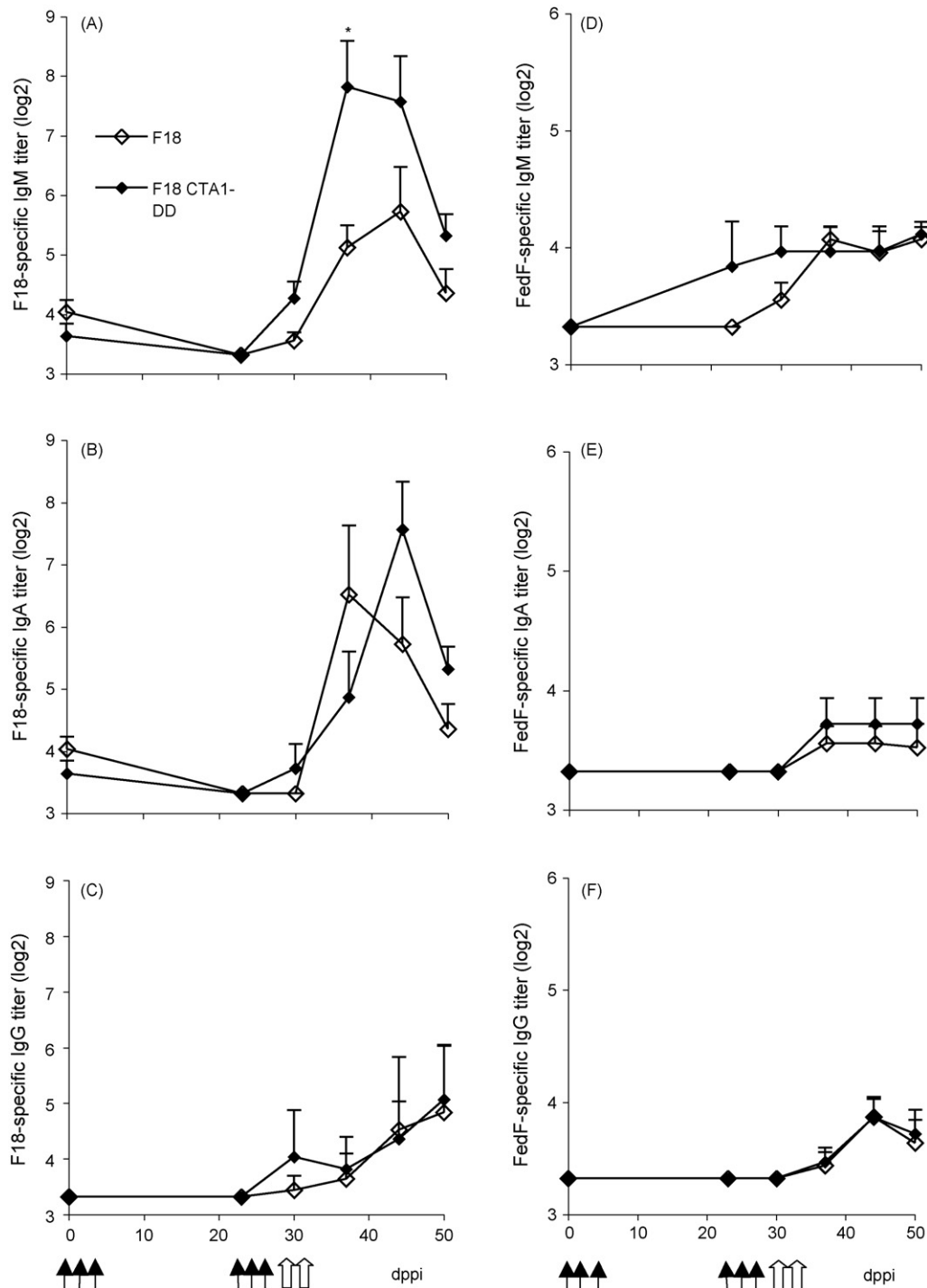


Fig. 3. Mean F18-specific IgM (A), IgA (B) and IgG (C) serum antibody titers ( $\pm$ S.E.M.) and FedF-specific IgM (D), IgA (E) and IgG (F) serum antibody titers ( $\pm$ S.E.M.) of piglets ( $n = 5$  per group) nasally immunized with 1 mg F18 (F18 group) or 1 mg F18 + 100  $\mu$ g CTA1-DD (F18 + CTA1-DD group). \*Significant difference ( $p < 0.05$ ). Black arrow, immunization; white arrow, F18<sup>+</sup> VTEC challenge.

adjuvant is reported as a powerful adjuvant for mucosal immunizations in mice (Gerber et al., 2001; Choi et al., 2002) and other animals (Burr et al., 2005; Jones et al., 2006). In pigs, LT(R192G) is shown to be an effective

adjuvant for nasal immunization (Yuan et al., 2000, 2001), but has not yet been used in oral immunization. The efficacy of bacterial enterotoxins as adjuvants for oral immunizations of mice and pigs seems to be

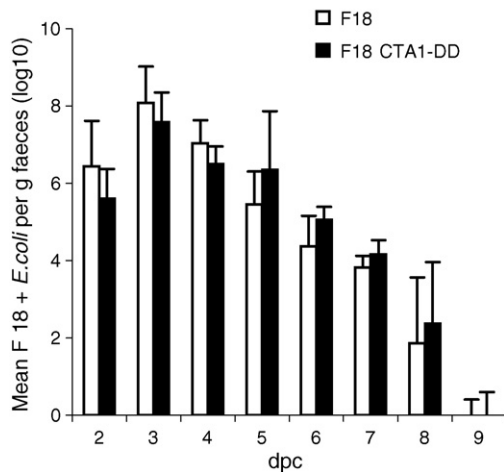


Fig. 4. Mean faecal F18<sup>+</sup> *E. coli* excretion (log<sub>10</sub>) per gram faeces (±S.E.M.) of piglets ( $n = 5$  per group) nasally immunized with 1 mg purified F18 fimbriae (F18 group) or 1 mg purified F18 fimbriae with 100 µg CTA1-DD (F18 + CTA1-DD group).

different. Oral immunization of mice with an antigen in the presence of cholera toxin (CT) results in the induction of an antigen-specific immune response (Clarke et al., 1991; Pierre et al., 1992). In pigs, the adjuvant activity of CT to orally co-administered heterologous antigens seems to depend on the antigen characteristics and the immunization schedule (Foss and Murtaugh, 1999; Verdonck et al., 2005). In a previous experiment with a similar immunization protocol (Verdonck et al., 2005), we observed that oral co-administration of 50 µg CT and 4.8 mg HSA-HSA conjugates induced a weak HSA-specific IgG response 21 dpi. Targeting HSA to the intestinal mucosae by the construction of F4-HSA conjugates and co-administration with CT, improved the induction of a HSA-specific immune response. In an oral immunization experiment of Foss and Murtaugh (1999), CT was only effective as mucosal adjuvant when keyhole limpet hemocyanin (KLH) was targeted to the intestinal mucosae (via binding of KLH to CT-B). Therefore, it would be interesting to analyse the mucosae-binding potential of purified F18 following oral administration. F4 is a very stable protein that will remain largely biologically active following transit through the stomach (Snoeck et al., 2004), but the influence of low pH and intestinal enzymes on F18 fimbriae is not analysed yet. Perhaps, only the 30 mg dose of F18 fimbriae resulted in substantial binding of F18 fimbriae to the F18R and consequent adjuvanticity of LT(R192G).

As oral immunization of newly weaned piglets with F18 fimbriae in the presence of LT(R192G) was not effective to prevent F18<sup>+</sup> *E. coli* colonization, a second

experiment was performed in which piglets were immunized intranasally. To mimic the field situation, piglets were already immunized with F18 fimbriae at the age of 1 week, boosted at weaning and challenged 1 week later, a time point when infections are regularly seen in the field (Fairbrother et al., 2005). The piglets were immunized nasally since uptake of milk during suckling can interfere with binding of fimbriae to their specific receptor and consequently impair the induction of fimbriae-specific antibodies (Snoeck et al., 2003). In addition, lymphocytes sensitized in the nasal-associated lymphoid tissues can relocate to distant effector sites such as the lamina propria (Brandtzaeg et al., 1999). Indeed, Yuan et al. (2000) reported the induction of IgA, IgM and IgG antigen-specific antibody secreting cells (3–37 per  $5 \times 10^5$  mononuclear cells) in the intestine following nasal immunization of pigs, despite the dichotomy in bronchial and intestinal immune responses (VanCott et al., 1994; Holmgren and Czerkinsky, 2005). Furthermore, CTA1-DD was used as adjuvant since it is non-toxic and its adjuvant activity following nasal administration was reported to be comparable to that of unmodified CT (Agren et al., 1998; Eriksson et al., 2004).

In the present study, CTA1-DD had only a slight adjuvant effect evidenced by the weak antibody response following the booster immunization and the significantly higher IgM response in the F18 + CTA1-DD group. Furthermore, the F18-specific serum antibody response increased faster and reached higher titers in both intranasally groups as compared to the response seen in an experimental infection of non-immunized piglets of the same age (Verdonck et al., 2002). These data suggest that intranasal immunization with F18 fimbriae primed a fimbriae-specific antibody response. However, the number of fimbriae-specific lymphocytes in the small intestine was probably low since boosting of the FedF-specific response was not observed following the challenge infection, even though there was a slight rise in the FedF-specific IgM response by the immunization. This could explain why there was no significant difference observed in F18<sup>+</sup> *E. coli* excretion following challenge of piglets immunized intranasally with F18 fimbriae in the presence or absence of CTA1-DD and why the excretion followed the pattern seen in non-immune pigs (Verdonck et al., 2002).

The induced fimbriae-specific immune response is directed against the F18 major fimbrial subunit FedA and in a limited extent against the minor adhesive subunit FedF. Immunizations with type 1 fimbriae induced a non-protective immune response, mainly directed against the major subunit FimA (Levine et al.,



1982). However, intramuscular vaccination of mice and monkeys with recombinant minor adhesive FimH subunits protects them against uropathogenic *E. coli* challenge (Langermann et al., 1997, 2000). In addition, intramuscular vaccination of chickens with the minor P pilus subunit and adhesin PapGII induced antibodies that could inhibit avian pathogenic *E. coli* binding (Vandemaële et al., 2006). Recently, oral immunization with recombinant adhesins is reported to induce a partial protection (Seo et al., 2002; Verdonck et al., 2004, 2005). The F18 fimbrial adhesin FedF is conserved among F18<sup>+</sup> *E. coli* field isolates from different places around the world (Tiels et al., 2005). Therefore, oral immunization of piglets with recombinant FedF or its lectin domain (Smeds et al., 2003) has to be considered.

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